



## Florida State University

### Polyclonal Antibody Production Protocol - Rabbits

While many species may be used for polyclonal antibody production (chickens, goats, guinea pigs, mice, sheep, rats, rabbits, horses and hamsters), the rabbit is the most commonly used for reasons of historical antecedents, cost-benefit ratio and ease of handling. The selection of species should be based upon several factors:

- Amount of antibody required. Larger species (sheep, horses, goats) are often chosen when large quantities of antibody are desired. Rabbits are a compromise between farm animals and rodents or chickens.
- Phylogenetic relationship between antigen donor and antigen producer. In general, the more distant the phylogenetic relationship the more potential for a high antibody titer.
- Required characteristics of the antibody. Such include the class, isotype and complement fixing nature of the antibodies to be produced.
- The intended use of the antibody. For example the antigen-binding antibody may need to be from a different species than the secondary antibody in an ELISA assay.
- Selection of strain within a species may be important with regard to differences in the MHC complex or immune regulatory mechanisms.

Young rabbits (2.5-3.0 kg; 10-16 weeks of age) should be used. At this age maternal IgG antibodies have declined to undetectable levels while the individual's immune system is approaching near adult levels. Older rabbits are not as useful for antibody production as immune function peaks at puberty and the ability to respond to new antigens declines with age. Female rabbits are more often used due to their docility. There are also reports that females (of many species) are sensitive to lower doses of antigen and may have significantly higher and more prolonged responses to immunization than males.

When calculating the number of animals to request, 2-3 animals per antigen is recommended to account for response failure, based upon previous reports. This is because there may be variability of the antigenic response in different individuals. Using more than one animal allows a more diverse response in terms of quantity of antibodies, specificity, and affinity. To comply with the 3Rs (reduction, replacement, refinement), if possible a sequential immunization approach should be used wherein if the first rabbit produces a high antibody titer then there is no need for a second or third animal.

Antigens used should be clean of contaminants and avoid extremes of pH. Extraneous microbial contamination, protein contaminants, chromatographic by-products (such as polyacrylamide gel) or chemical contamination (i.e., SDS, urea, acetic acid and solvents) may lead to a low titer of the desired antibody. In addition, contaminants and pH extremes can contribute to inflammatory reactions detrimental to the health of the animal. Sterilization by filtration through a low binding 0.22 micron filter (i.e., cellulose acetate) should be done whenever possible. Antigen prepared by gel electrophoresis should be either (1) eluted, lyophilized, ground to a fine powder, and re-suspended in sterile saline or (2) transferred to nitrocellulose paper, trimmed and cut into fine pieces.

Injections must only be done with sterile needles and syringes. Freund's should be used with glass syringes. The amount of antigen to be injected will vary, but in general for rabbits use of 50-1000 ug is recommended. Booster doses are usually one half to equal the priming dose.

The ideal adjuvant should be capable of helping to induce a high titer of high avidity antibodies while having few to no side effects. Unfortunately there is no single adjuvant agent ideal for use with all antigens or in all situations. Each available product has advantages and disadvantages. The most commonly used adjuvants at FSU are RIBI and Freund's complete/incomplete. Please review the document 'Antibody Production - Adjuvants' for further information. In addition to adjuvant selection, consider whether a carrier protein should be used as a conjugate to enhance the immune response. While many antigens are immunogenic by themselves, small molecular weight peptides (<15 KD) and non-protein or synthetic peptides may be poorly antigenic. These antigens may need to be coupled to a carrier protein to increase their immunogenicity. Peptides larger than 25-30 KD probably do not need conjugation. Common conjugating proteins include keyhole limpet hemocyanin (KLH), bovine serum albumin, thyroglobulin and ovalbumin. In some cases, enhanced immunogenicity is achieved by covalently linking immunogens to liposomes prior to injection. The choice of carrier should be based on its own ability to stimulate the immune response as well as its ability to be adequately coupled to the desired antigen.

For further information on preparation of antigens, refer to the UC Berkley ACUC [Guidelines for Polyclonal Antibody Production in Laboratory Animals](#).

For scientific reasons, the reuse of rabbits in other immunization protocols is usually inappropriate. For humane reasons, re-immunization with Freund's complete adjuvant is inappropriate and only permitted with ACUC approval. However, because immunization and bleeding are non-invasive and low stress procedures, antibody producing rabbits may be used in other experiments not involving antibody production.

It is recommended that investigators collect a pre-immunization blood sample. On occasion, rabbits may have closely related antibodies to something in the rabbit's environment or their feed which may be similar to the antigen under investigation.

Following completion of any and all procedures, the individual animal's record must be updated. Observations to include: date, antigen and amount, adjuvant, route, sites, total volume, blood collection volume, sedation or anesthetics used, blood collection site. Observations of post procedural complications (not eating, localized swellings, ulcers, etc.) should be made as soon as noticed. Any treatment for such must be recorded in the animal's record.

### **Standard Polyclonal Antibody Production Schedule for Rabbits:**

*Note: Inappropriate handling of rabbits can result in life threatening injuries. Training on how to handle rabbits and to perform injection and blood collection procedures is available from LAR. Project personnel should demonstrate their ability to perform these procedures to the satisfaction of the attending veterinarian or a qualified individual designated by the attending veterinarian.*

*Wear appropriate PPE (lab coat, gloves and possibly protective ear wear) when handling rabbits to avoid induction of animal allergies.*

*Remove the rabbit from its cage and place it on a solid surface that is rough enough for the rabbit to*

*feel comfortable. Laboratory benches should be avoided as the rabbit will constrict its muscles on any smooth surface, however wrapping the rabbit in a towel is often sufficient and this may serve as a restricting device. Use of the rabbit restrainer may be necessary. In general, routine sedation is not necessary for antigen injections but is recommended for blood collection (see below).*

*In general, boosters are recommended to be given at 2- 4 week intervals. Blood collections should be performed 7-10 days following a booster injection for maximum results, depending upon the titer response curve generated per antigen or animal.*

Day 0	Pre-immunization bleed + initial antigen injection
Day 14 - 21	First antigen booster
Day 28 - 35	Second antigen booster
Day 35 – 42	Test bleed
Day 42 – 56	Third antigen booster
Day 49 – 66	Blood collection

Alternations of antigen injections and blood collections may be done at additional times as needed for antibody production with the exception that if an animal fails to produce a satisfactory titer after the fourth booster it should be considered unsuitable for the antigen and taken out of the study. Variations differing from the above recommendations must be described in the individual investigator's protocol.

### **Post-injection monitoring**

- Observe animals for a minimum of 15 minutes after the final injection or blood collection that day. Contact LAR staff immediately if an abnormal reaction is noticed.
- Animals must be checked at least three times per week for 4 weeks after immunization.
- If complications are noted, animals must be checked daily.
- Investigators, veterinary staff and animal technicians should monitor rabbits for signs of pain or distress as well as swelling, abscesses or ulceration at the injection sites.
- Staff (investigator or animal care) should contact LAR veterinarians if any of the above signs are noticed in an animal.

### **Intradermal injections for rabbits**

1. Restrain the rabbit in an appropriate manner.
2. Intradermal injections for rabbits should be 0.05 ml or less per injection site; maximum of 20 sites for a total per injection session not to exceed 1 ml.
3. Do not place injections in sites that are used for grasping or physical restraint. Sites should be far enough apart to prevent coalescence of the local inflammatory reaction.
4. Injections sites must be clipped and disinfected with 70% alcohol before injections are made.
5. To give an ID injection a 25-gauge needle attached to a tuberculin syringe is inserted at a 10-15° angle, bevel up, just under the epidermis.
6. Verify that the needle is not in a blood vessel by pulling back on the plunger. If no blood appears in the needle hub, then the needle is not in a blood vessel.
7. Depress the plunger to inject the desired amount. An intradermal injection will cause an obvious small wheal or bump to form if the needle has been placed correctly. Following a few seconds pause, the needle is withdrawn. Do not continue to apply pressure to nor draw back on the plunger to avoid contamination of the needle track with the antigen/adjuvant mixture.
8. Repeat this procedure until the total volume has been delivered.

9. Check to make sure there is no bleeding from any injection site. If bleeding is noted, apply pressure for 1-3 minutes until stopped. Return rabbit to cage.
10. Follow post-injection monitoring procedures above.

### **Subcutaneous injections for rabbits**

1. Restrain the rabbit in an appropriate manner.
2. Subcutaneous injections for rabbits should be 0.2 ml or less per injection site; maximum of 10 sites for a total per injection session not to exceed 2 ml.
3. Do not place injections in sites that are used for grasping or physical restraint. Sites should be far enough apart to prevent coalescence of the local inflammatory reaction. Prep areas with 70% alcohol being injecting.
4. Starting on one side near the dorsal shoulders, skin is pinched between the thumb and a finger, pulled away from the body to form a tent and a needle (20 gauge or smaller) is inserted into the space that has been created.
5. Verify that the needle is in the subcutaneous space and is not inserted into muscle or the body wall.
6. Verify that the needle is not in a blood vessel by pulling back on the plunger. If no blood appears in the needle hub, then the needle is not in a blood vessel.
7. Depress the plunger to inject the desired amount. Following a few seconds pause, the needle is withdrawn. Do not continue to apply pressure to nor draw back on the plunger to avoid contamination of the needle track with the antigen/adjuvant mixture.
8. Repeat this procedure at other sites of the body, rotating through all four quadrants (right front, right rear, left front, left rear) until total volume has been delivered.
9. Check to make sure there is no bleeding from any injection site. If bleeding is noted, apply pressure for 1-3 minutes until stopped. Return rabbit to cage.
10. Follow post-injection monitoring procedures above.

### **Intramuscular injections for rabbits**

1. Intramuscular injections are not recommended for CFA/IFA in rabbits but may be used with appropriate justification and approval by the ACUC.
2. Restrain the rabbit in an appropriate manner.
3. Intramuscular injection sites are limited. For rabbits, an injection of 0.3 ml into one hindlimb may be used. Alternatively, the lumbar muscles may be used with 0.3 ml injected per side.
4. Clean the injection site with 70% alcohol prior to injection.
5. Use a 23 gauge needle. Insert the needle into the body of the muscle. Verify that the needle is not in a blood vessel by pulling back on the plunger. If no blood appears in the needle hub, then the needle is not in a blood vessel.
6. Depress the plunger to inject the desired amount. Following a few seconds pause, the needle is withdrawn. Gently massage the injected area.
7. Repeat this procedure on the other side if doing lumbar muscle injections.
8. Check to make sure there is no bleeding from any injection site. If bleeding is noted, apply pressure for 1-3 minutes until stopped. Return rabbit to cage.
9. Follow post-injection monitoring procedures above.

Note: The IM route may be combined with the SC route in certain regimens, however do not exceed a total volume of 2 ml antigen/adjuvant divided as noted above.

### **Intravenous injections for rabbits**

May never be used with Complete Freund's adjuvant.

Only experienced personnel should use this route.

Place rabbit in a snug restraining device. Sedation or topical anesthesia is recommended but not always necessary for experienced personnel. IF sedation or anesthesia is used, this should be administered and allowed to take effect before restraining the animal for blood collection.

1. Intravenous injections are normally performed in the marginal ear vein. A normal iv injection is 500-1000 microliters. With the ear out straight, the vein is on the edge of the ear. By pressing lightly on the base of the ear, the return of the blood to the body can be restricted and the vein will stand out strongly. Some individuals may wish to pluck the hair over the injection site. Clean the injection area with 70% alcohol being injecting the mixture. Use of topical irritants to dilate the blood vessels is not allowed.
2. Insert 22-25-gauge needle into the vein. The rabbit may twitch or move abruptly in response to being pricked by the needle, so make sure the animal is safely restrained. When the needle appears to be in the vein, gently pull back on the plunger. If blood appears in the needle hub, slowly inject the contents of the syringe. If no blood is seen, another location is selected.
3. After a short pause the needle is removed. A cotton or sterile gauze is placed over the injection site as the needle is removed. Once the syringe and needle are clear, pressure is applied to the injection site. Holding the site tightly for a few seconds will stop any bleeding.
4. Wait 1-3 minutes to verify that any bleeding from the injection site will not occur and no adverse reactions occur.
5. Return rabbit to its cage.
6. Follow post-injection monitoring procedures above.

For most laboratory animals the intravenous injection (iv) is a practical method of delivering a secondary or later boost. It is seldom used for primary injection. Antigen delivered directly in the blood stream will be processed rapidly by the reticuloendothelial system, primarily in the liver, lungs, and spleen. Normally, this procedure causes little pain and distress other than that accompanying the needle prick. However, iv injection does carry a low but potentially serious risk to the host animal, as it can induce pulmonary embolisms or lethal anaphylactic shock in sensitized animals. Always consult with LAR and/or other experts before injecting any novel substance iv. Any toxic material present in the inoculum, such as bacterial endotoxins, is particularly dangerous when delivered iv. Physiological buffers and salt solutions should be used if at all possible. Detergent concentration should never be allowed to exceed 0.1% for ionic detergents or 0.2% for non-ionic.

### **Test bleed on rabbits – marginal ear vein**

1. Restrain the rabbit in an appropriate manner. In general, a restraint device will be used rather than a person merely holding the animal. Sedation or topical anesthesia is recommended but not always necessary for experienced personnel. If sedation or anesthesia is used, this should be administered and allowed to take effect before restraining the animal for blood collection.
2. The marginal vein is found on the inner edge of the dorsal surface of the ear and should be visible. A patch over the vein about two-thirds of the distance from the head to the tip of the ear is gently plucked clean of hair or shaved. Prep the area with 70% alcohol or chlorhexidine solution.
3. If the marginal vein is not visible or easily found, the ear may be gently heated under a low wattage lamp or by application of a warm compress. Use of topical irritants such as xylene to dilate the blood vessel is not permitted as it may cause skin damage.

4. Blood collection is done only with a sterile needle and syringe or butterfly catheter and syringe. The ear is held out horizontally and stabilized. Penetrate the vein with the needle bevel up. Only a small portion of the needle is inserted parallel above the vessel with the tip directed into the lumen along with the longitudinal axis. Sampling of blood from the vein should be performed as close to the base of the ear as possible. Additional attempts can be made distally toward the ear tip for the vein. Proper insertion of the needle into the vein is the most important part of the procedure. While guidance and training can be provided, individuals should understand that practice is necessary in order to achieve proficiency.
5. Withdraw the amount of blood desired but bear in mind that only small volumes (less than 3 ml) will be obtainable from the marginal ear vein due to its size. If larger amounts are necessary, then blood should be collected from the central ear artery. Also keep in mind that rabbits are capable of constriction of their peripheral blood vessels, particularly when distressed. Use of a topical anesthetic such as EMLA cream or lidocaine jelly helps minimize this reaction.
6. Following completion of blood withdrawal, remove the needle and cover the vein penetration site with a cotton ball. Apply gentle pressure to the site for up to 2 minutes to ensure adequate hemostasis.
7. Verify that no further bleeding is occurring and return rabbit to its cage.
8. Follow post-injection monitoring procedure above.

*Note: Blood collection from rabbit ears by transecting or cutting the vein s not permitted. Never use a scalpel or needle bevel to cut the vessels. Always insert a needle into the vessel.*

#### **Blood collection rabbits – central ear artery**

1. Restrain the rabbit in an appropriate manner. In general, a restraint device will be used rather than a person merely holding the animal. Sedation or topical anesthesia is highly recommended but not always necessary for experienced personnel. If sedation or anesthesia is used, this should be administered and allowed to take effect before restraining the animal for blood collection. Recommendation for anesthesia is (1) 0.2-1.0 mg/kg acepromazine SC + topical 2% lidocaine HCl jelly or EMLA cream or (2) 1.0 mg/kg acepromazine + 1.0 mg/kg butorphanol SC.
2. The central artery is easily located on the dorsal surface of the ear and should be visible. Plucking or shaving is not necessary but may be done if desired. Prep the area with 70% alcohol or chlorhexidine solution.
3. Sedation or anesthesia should dilate the artery for ease of collection. If not using sedation or anesthesia and the artery appears to be small or constricted, the ear may be gently heated under a low wattage lamp or by application of a warm compress. Use of topical irritants such as xylene to dilate the blood vessel is not permitted as it may cause skin damage.
4. Blood collection is done only with a sterile needle and syringe, butterfly catheter and syringe or vacutainer needle and tubes. The ear is held out horizontally and stabilized. Penetrate the artery with the needle bevel up. Only a small portion of the needle is inserted parallel above the vessel with the tip directed into the lumen along with the longitudinal axis. Sampling of blood from the artery, unlike the vein, should be performed as close to the tip of the ear as possible. Additional attempts can be made closer to the base of the ear if needed. Proper insertion of the needle into the artery is the most important part of the procedure. While guidance and training can be provided, individuals should understand that practice is necessary in order to achieve

proficiency. Keep in mind that rabbits are capable of constriction of their peripheral blood vessels, particularly when distressed. Use of a topical anesthetic such as EMLA cream or lidocaine jelly helps minimize this reaction. Patience and gentle massage may help the artery to re-dilate.

5. Withdraw the amount of blood desired. For a single blood collection, up to 15% of the total blood volume based upon the animal's body weight may be collected once a month. If weekly collections are necessary, the maximum volume will be 7.5% of the total blood volume based upon the animal's body weight. Routine weekly collections or collections exceeding the previous values will require routine evaluation of the rabbit's packed cell volume (PCV). \*See example calculations below.
6. Following completion of blood withdrawal, remove the needle and cover the penetration site with a cotton ball. Apply gentle pressure to the site for up to 2 minutes to ensure adequate hemostasis.
7. Verify that no further bleeding is occurring and return rabbit to its cage.
8. Follow post-injection monitoring procedure above.

Example – determination of blood collection volume:

(a) Single collection per month: 4 kg rabbit

$50\text{-}60\text{ ml/kg} \times 4\text{ kg body weight} = 200\text{-}240\text{ ml total blood volume} \times 0.15 = 30\text{-}36\text{ ml maximum blood that can be collected once/month}$

(b) weekly collection of 4 kg rabbit

$50\text{-}60\text{ ml/kg} \times 4\text{ kg body weight} = 200\text{-}240\text{ ml total blood volume} \times 0.075 = 15\text{-}18\text{ ml maximum blood that can be collected weekly.}$

*Note: The Norwegian offers video Reference Centre for Laboratory Animal Science and Alternatives has slide/video demonstration of this [technique](#) on line.*

### **Terminal Procedure – Intracardiac blood collection**

*Note: Intracardiac puncture for large volume blood collection is limited to terminal procedures only and is performed under general anesthesia. It is not an acceptable method for routine or repeat blood collection.*

1. Anesthetize the rabbit with xylazine (7-10 mg/kg) + ketamine (40-50 mg/kg) IM.
2. Once the animal has been verified to be in a surgical plane of anesthesia, lay animal on its back or side, depending upon the phlebotomist's preference.
3. Prep the area with 70% alcohol or chlorhexidine and insert needle at base of sternum (under the Xiphoid) at a 30-45° angle just lateral to the midline (rabbit's left side) or the needle can be inserted into the lateral thoracic region toward the area of maximal heart beat between the ribs of the rabbit's left side midway between its sternum and back under the left elbow.
4. If using a syringe, slowly aspirate the desired amount, changing syringes as needed. If using vacutainer tubes push the tube onto the outer needle once placement is correct. The blood should flow quickly into the container. Collect blood until no further blood is able to be obtained; death occurs via exsanguination.
5. Verify that heart has stopped. If it is still beating, euthanize with 100 mg/ml barbiturate euthanasia solution intracardiac. This will ensure that the heart has stopped prior to disposal.
6. Wait at least 15 minutes, then confirm that death has occurred before putting the carcass into a bag and placing it into a medical waste disposal bin in the morgue freezer.

*Note: The Norwegian offers video Reference Centre for Laboratory Animal Science and Alternatives has slide/video demonstration of this [technique](#) on line.*

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